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Endoplasmic Reticulum and *cis*-Golgi Localization of Human T-Lymphotropic Virus Type 1 p12^{I}: Association with Calreticulin and Calnexin

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Human T-lymphotropic virus type 1 (HTLV-1) is a complex retrovirus encoding regulatory and accessory genes in four open reading frames (ORF I to IV) of the pX region. We have demonstrated an important role of pX ORF I expression, which encodes p12^{I}, in establishment of HTLV-1 infection in a rabbit model and for optimal viral infectivity in quiescent primary lymphocytes. These data indicated that p12^{I} may enhance lymphocyte activation and thereby promote virus infection. To further define the role of p12^{I} in cell activation, we characterized the subcellular localization of p12^{I} in transfected 293T cells and HeLa-Tat cells by multiple methods, including immunofluorescence confocal microscopy, electron microscopy, and subcellular fractionation. Herein, we demonstrate that p12^{I} accumulates in the endoplasmic reticulum (ER) and *cis*-Golgi apparatus. The location of p12^{I} was unchanged following treatments with both cycloheximide (blocking de novo protein synthesis) and brefeldin A (disrupting ER-to-Golgi protein transport), indicating that the protein is retained in the ER and *cis*-Golgi. Moreover, using coimmunoprecipitation assays, we identify the direct binding of p12^{I} with both calreticulin and calnexin, resident ER proteins which regulate calcium storage. Our results indicate that p12^{I} directly binds key regulatory proteins involved in calcium-mediated cell signaling and suggest a role of p12^{I} in the establishment of HTLV-1 infection by activation of host cells.

Human T-lymphotropic virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia and lymphoma and appears to initiate a variety of immune-mediated disorders, including the chronic neural degenerative disease HTLV-1-associated myelopathy/tropical spastic paraparesis (16, 44). As a complex retrovirus, HTLV-1 contains the common retroviral genes *gag*, *pol,* and *env,* as well as several regulatory and accessory genes. These regulatory and accessory genes are present in four different open reading frames (ORFs) in the pX region between *env* and the 3′ long terminal repeat (LTR) (10, 15, 39, 40). ORFs IV and III encode the regulatory proteins Tax and Rex, respectively, which have been extensively characterized. Tax is a 40-kDa nuclear-localizing protein that increases viral transcription from the HTLV-1 LTR as well as a number of cellular genes involved in host cell proliferation (17, 30, 34). Rex is a 27-kDa nucleolar-localizing protein that acts at the posttranscriptional level by promoting the cytoplasmic accumulation of unspliced and singly spliced viral RNA (29).

Recent studies have provided important new data that indicate a role of the highly conserved ORF I-encoded protein p12^{I} in HTLV-1 infection. ORF I mRNA has been detected in HTLV-1-infected cells derived from patients with both adult T-cell leukemia and lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis and from asymptomatic carriers (3, 5, 9, 10). Moreover, recombinant p12^{I} is recognized by sera from naturally infected humans and experimentally infected rabbits (14). Peptides derived from amino acid sequences unique to ORF I-encoded proteins are recognized by cytotoxic T lymphocytes isolated from infected subjects, indicating the chronic production of these proteins during HTLV-1 infection (37). Members of our group have demonstrated that the selective ablation of ORF I mRNA dramatically decreases the infectivity of ACH, an infectious molecular clone of HTLV-1, in a rabbit model of infection (12). Additionally, we also demonstrated that ORF I expression is required for optimal viral infectivity in quiescent primary lymphocytes, suggesting a role of p12^{I} in T-lymphocyte activation (1).

These data imply a functional role of pX ORF I-encoded proteins in host cell activation. HTLV-1 p12^{I} is a small hydrophobic protein and has distant homology with the bovine papillomavirus E5 protein (17). The viral protein contains four minimal proline-rich SH3 domain binding motifs (PXXP), which are commonly present in cellular proteins involved in regulating signal transduction. The protein associates with the 16-kDa subunit of the vacuolar H^{+} ATPase, enhances E5 transforming ability (18), and binds to the interleukin 2 receptor β and γ chain (33). Moreover, Koralnik et al. (25, 26) have observed by indirect immunofluorescence assays that p12^{I} is predominantly found in the perinuclear region of HeLa-Tat

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cells and predicted that p12^{I} is expressed in cellular endomembranes. These studies together with the predicted structure motifs of p12^{I} imply a functional role for this protein in modulating cellular signals. Using subcellular fractionation, immunofluorescence assay, and confocal and electron microscopy, we demonstrate that the majority of p12^{I} is enriched in the endoplasmic reticulum (ER) and *cis*-Golgi apparatus. Proteins that are expressed in the ER achieve their specific localization by direct retention or by retrieval from cellular compartments through recognition of specific cellular motifs. To test whether p12^{I} maintained its localization by retention or retrieval, we used cycloheximide to block protein synthesis and tested for the expression of the protein over a 24-h period. The typical perinuclear localization of p12^{I} did not change following either cycloheximide treatment alone or cycloheximide plus brefeldin A (BFA) cotreatment, indicating that p12^{I} is retained in the ER and *cis*-Golgi compartment. The amino acid sequence of p12^{I} does not contain typical ER retention motifs. Therefore, the retention of p12^{I} in the ER is likely related to other structural features of the protein. Using serially deleted p12^{I} expression vectors, we demonstrated that two regions containing predicted transmembrane domains (amino acids [aa] 1 to 47 and aa 47 to 99) are independently sufficient for the localization of p12^{I}. Furthermore, p12^{I} colocalized with and bound calreticulin and calnexin, resident ER proteins important for calcium storage and release from the ER. Our data suggest a potential mechanism for p12^{I} in calcium-mediated cell activation to enhance HTLV-1 replication.

MATERIALS AND METHODS

Cell lines and plasmids. The 293 cell line is a human kidney embryonic cell line (catalog number 1573, American Type Culture Collection). 293T is the 293 cell line which stably expresses the simian virus 40 (SV40) T antigen (obtained from G. Franchini, National Institutes of Health). HeLa-Tat is a human cervical carcinoma cell line, HeLa, which stably expresses human immunodeficiency virus type 1 Tat protein. This cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health, from Barbara Felber and George Pavlakis (HLtat, catalog number 1293). These three cell lines were maintained in Dulbecco's modified Eagle medium (supplemented with 10% fetal bovine serum, 100 μg of streptomycin plus penicillin/ml, and 2 mM L-glutamine [Gibco]). The R49 cell line (13) is a rabbit peripheral blood mononuclear cell line which was stably transformed with ACH, an infectious molecular clone of HTLV-1 (24). R49 cells were maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum, 100 μg of streptomycin plus penicillin/ml, and 2 mM L-glutamine (Gibco). The pMEp12^{I} plasmid contains influenza virus hemagglutinin (HA) epitope-tagged p12^{I} sequence in the pME vector, which is driven by a hybrid promoter (SRα) containing the SV40 early promoter and the R region of the HTLV-1 LTR (33, 41). The pLEGFP-C1 plasmid is a retroviral green fluorescent protein expression vector (Clontech). The full-length p12^{I} sequence was PCR amplified from the ACH plasmid and directionally subcloned into the pLEGFP-C1 plasmid to construct pLEGFPp12^{I}. The pBCp12^{I} plasmid was created by subcloning p12^{I}-encoding sequence to the C terminus of a glutathione *S*-transferase (GST) sequence in the pBC vector (6), a mammalian GST expression vector driven by the SV40 promoter (obtained from C. Kredinger, Institute de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/LGME-INSERM, Strasbourg, France).

Construction of serial deletion mutants of p12^{I}. Serial deletion mutants of p12^{I}, C-terminally tagged with HA, were subcloned into the pME vector in two steps. Eight deletion mutants were first subcloned into the pME vector at *Eco*RI and *Not*I sites by PCR-mediated directional subcloning. The HA epitope was then synthesized as oligonucleotide and ligated at the C termini of all the deletion mutants using *Not*I and *Xba*I sites. The following primers were used in the PCRs to amplify the different regions of the p12^{I} encoding sequence.

p12^{I} 15-99 (5′, ATATGAATTCATGACGGCGCTCCTGCTC; 3′, ATATGCGGCCGCTTAGAAGAGGAAAGCCGC); p12^{I} 32-99 (5′, AAATTTGAATTCATGCTCCGCCCGCCTCCT; 3′, ATATGCGGCCGCTTAGAAGAGGAAAGCCGC); p12^{I} 48-99 (5′, ATAGAATTCATGATACTCAGCAATCTGCTT; 3′, ATATGCGGCCGCTTAGAAGAGGAAAGCCGC); p12^{I} 1-86 (5′, ATATGAATTCATGCTGTTTCGCCTTCTC; 3′, ATTGCGGCCGCGGGGAGAAAGCGCCACCTCGC); p12^{I} 1-47 (5′, ATATGAATTCATGCTGTTTCGCCTTCTC; 3′, ATTGCGGCCGCTTGAAAAGGAAGGAAGAGGAG); p12^{I} 15-86 (5′, ATATGAATTCATGACGGCGCTCCTGCTC; 3′, ATTGCGGCCGCGGGGAGAAAGCGCCACCTCGC); p12^{I} 15-69 (5′, ATATGAATTCATGACGGCGCTCCTGCTC; 3′, ATTGCGGCCGCGCTGAGGAGAAGAGGAAGCGA); p12^{I} 15-47 (5′, ATATGAATTCATGACGGCGCTCCTGCTC; 3′, ATTGCGGCCGCTTGAAAAGGAAGGAAGAGGAG).

The HA oligonucleotides included the sense oligonucleotide GGCCGCGTACCCATACGATGTTCCAGATTACGCTAGCTTGGCCGC GGCTTTCCTCTTCTAAT and the antisense oligonucleotide TAGATTAGAAGAGGAAAGCCGCGGCCAAGCTAGCGTAATCTGGAACATCGTATGGGTACGC. Sanger sequencing was used to confirm all of the inserted p12^{I} nucleotide sequences and to ensure that the sequences were in-frame.

Immunoblot assay. 293T cells were seeded at approximately 40% confluence in 10-cm-diameter tissue culture dishes 1 day before transfection. Cells were transfected with 10 μg of pMEp12^{I}, pME, or deletion mutants of p12^{I} using Lipofectamine Plus (Gibco). Transfected 293T cells were lysed at 48 h after transfection in Triton X-100 buffer [1% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), with protease inhibitor 20-μg/ml leupeptin, 20-μg/ml aprotinin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), and 1 mM sodium orthovanadate]. The protein concentration from all lysates was measured using a Micro BCA protein assay kit (Pierce). Forty micrograms of each lysate was separated by SDS–10% polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose membranes (Millipore), the expression of pMEp12^{I} and that of the deletion mutants of p12^{I} were detected with a mouse monoclonal anti-HA antibody (1:1,000; Babco), followed by incubation with a secondary antibody conjugated to horseradish peroxidase (1:1,000; New England Biolabs). Bands were visualized by using a chemiluminescence detection system (New England Biolabs).

Immunocytochemistry. For indirect immunofluorescence assays, HeLa-Tat cells were seeded into chamber slides (Fisher Scientific) and were transfected with 4 μg of pMEp12^{I} or deletion mutants of pMEp12^{I} using Lipofectamine Plus. Two days posttransfection, cells were fixed for 15 min with 4% paraformaldehyde, followed by incubation with primary antibodies: mouse monoclonal anti-HA (1:200; Babco), rabbit polyclonal anticalreticulin (1:500; Affinity Bioreagents), or mouse monoclonal anti-adaptin γ (1:200; Sigma) for 1 h at room temperature in antibody dilution buffer (0.01 M sodium phosphate, 0.5 M NaCl, 0.5% Triton X-100, 2% bovine serum albumin). Cells were washed three times with phosphate-buffered saline (PBS) (Gibco) and were incubated with either fluorescein isothiocyanate-labeled anti-mouse antibody plus indocarbocyanine (Cy3)-labeled anti-rabbit or Cy3 labeled anti-mouse antibody (Jackson Immunogen) at room temperature for 1 h. To identify subcellular organelles, specific markers, including the Golgi marker, BODIPY TR ceramide, the mitochondrion marker, MitoTracker Orange, and the F-actin marker, rhodamine phalloidin, were used to costain with p12^{I} expression according to the manufacturer's protocol (Molecular Probes). Briefly, 5 μM BODIPY TR ceramide was added to the secondary antibody dilution buffer, and cells were stained at room temperature for 1 h. MitoTracker Orange (100 nM) was added to Dulbecco's modified Eagle medium, and live cells were stained for 30 min, followed by the indirect immunofluorescence detection of p12^{I} as described above. Rhodamine phalloidin (1:100) was added to the secondary antibody dilution buffer, and cells were costained for both F-actin and p12^{I}. The images were collected using the Lasersharp software program with a Bio-Rad MRC1024 confocal microscope.

BFA and cycloheximide treatments. To test the effect of BFA treatment on p12^{I} localization, pLEGFPp12^{I}-transfected HeLa-Tat cells were treated with 10 μg of BFA/ml for 30 min at 2 days posttransfection. The localization of GFPp12^{I} and that of adapter complex protein 1 (AP-1) were detected by direct and indirect immunofluorescence assays, respectively, as described above. For cycloheximide studies, 10 μg of cycloheximide (Sigma)/ml was added into pMEp12^{I}-transfected HeLa-Tat cell medium at 24 h posttransfection, and cells were treated for 0.5, 2, 4, 8, 12, or 24 h, followed by detection of p12^{I} accumulation by indirect immunofluorescence assay. For cycloheximide and BFA cotreatment, cells were incubated with 10 μg of BFA/ml for 30 min before cell fixation at different time periods of cycloheximide treatments, followed by indirect immunofluorescence assay to examine p12^{I} expression.

Electron microscopy. Electron microscopy was used to further identify the subcellular organelles expressing p12^{I}. HeLa-Tat cells were transfected with 15 μg of pMEp12^{I}. Cells were fixed with 2.5% paraformaldehyde and 0.5% glutaraldehyde at 48 h posttransfection, followed by dehydration and embedding in a commercial resin (LR White, Electron Microscopy Science). Embedded samples

were sectioned with an LKB Ultratome and were deposited on nickel grids. The grids were incubated with mouse monoclonal anti-HA antibody (1:50; Babco) at room temperature for 1 h, followed by incubation with the 10-nm colloidal gold-conjugated anti-mouse antibody (1:100; Amersham Pharmacia Biotech) for 30 min at room temperature. The samples were examined by electron microscopy (Phillips 300) at ×45,000 or ×53,000 magnification, and images were processed using standard photographic methods.

Membrane and cytosol fractionation. The membrane and cytosol fractions of pMEp12^{I}-transfected cells were separated to test the association of p12^{I} with the membrane. Transfected 293T cells were resuspended in 1 ml of homogenization buffer (200 mM HEPES [pH 7.5], 5 mM sodium pyrophosphate, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM AEBSF, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml, 1 mM sodium orthovanadate), followed by sonication and centrifugation at 11,000 rpm for 1 min. The supernatant was ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and the resulting cytosol fraction (the supernatant portion) was collected. The pellet was washed twice with PBS and resuspended in 1 ml of extraction buffer (20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM NaF, 1 mM AEBSF, 1 mM sodium orthovanadate), followed by shaking for 1 h at 4°C and centrifugation at 100,000 × *g* for 45 min. The supernatant was collected as the membrane fraction. The protein concentrations from both membrane and cytosol fractions were measured using the Micro BCA protein assay kit. The expression of p12^{I}, Ras-related GTP binding protein 1B (Rab1B), and Fas-associated death domain protein (FADD) in both membrane and cytosol fractions were tested by immunoblot analysis using mouse monoclonal anti-HA antibody, rabbit polyclonal anti-Rab1B antibody (Zymed), and mouse monoclonal anti-FADD antibody (Transduction Lab), respectively, as described previously (7, 27, 45).

Subcellular membrane fractionation. To identify the subcellular membranes that contain p12^{I}, gradient ultracentrifugation and fractionation were performed. Approximately 10^8 pMEp12^{I}-transfected 293T cells at 48 h posttransfection were resuspended in 3 ml of homogenization buffer (HB) (containing 10 mM HEPES [pH 7.4], 1 mM EDTA, 0.25 M sucrose, 20-μg/ml leupeptin, 20-μg/ml aprotinin, 1 mM AEBSF, and 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by 10 strokes with a Dounce homogenizer followed by 5 to 10 passages through a 27-gauge needle to obtain about 95% broken cells. Nuclei and unbroken cells were pelleted by centrifugation at 1,500 rpm for 10 min in an Eppendorf 5714R centrifuge. The postnuclear supernatant was centrifuged in an SW55 rotor (Beckman) at 65,000 × *g* for 1 h. The resulting pellet was resuspended in 0.8 ml of HB buffer. Discontinuous iodixanol (OptiPrep, 60% wt/vol; Gibco) gradients were prepared in an SW41 centrifugation tube as described previously (46). Briefly, iodixanol was diluted to 50% in HB buffer as the stock solution, which was then used to make various concentrations of iodixanol used in gradient experiments. Gradients were set up in a 13-ml centrifuge tube by underlaying solutions with defined percentages of iodixanol with a syringe and metal needle to create a gradient consisting from top to bottom of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, and 30% iodixanol. The resuspended pellet was loaded on top of the gradients and centrifuged in an SW41 rotor at 41,000 rpm for 2.5 h. The resulting gradient was collected as 1-ml fractions (total, 13 fractions). The fractions were solubilized by adding Triton X-100 to a final concentration of 1%, followed by immunoblot analysis to test for the expression of p12^{I}, the ER marker, calnexin, and the Golgi 58-kDa protein as described previously (4, 19, 38, 47).

Immunoprecipitation assay for calreticulin and calnexin binding. To investigate the association of p12^{I} with calreticulin and calnexin, 293T cells were transfected with 10 μg of pMEp12^{I}. Cells were lysed with Triton X-100 buffer. Cell lysates were precleared with both 30 μl of protein A Sepharose beads (Sigma) and 6 μl of normal rabbit serum for 6 h, followed by incubation with either 1:150 diluted rabbit polyclonal anticalreticulin (Affinity Bioreagents) or 1:150 diluted rabbit polyclonal anticalnexin (StressGene) overnight. The immune complex mixture was incubated with 30 μl of protein A Sepharose beads for 2 h. Beads were washed twice in Triton X-100 lysis buffer and boiled in SDS sample buffer, and the supernatants were separated by SDS-PAGE. The proteins were examined for p12^{I} expression by immunoblot assay using a mouse monoclonal anti-HA antibody (1:1,000; Babco). The endogenous expression of calreticulin and that of calnexin were tested by immunoblot assay using chicken polyclonal anticalreticulin (Affinity Bioreagents) and the same rabbit polyclonal anticalnexin described above. As an additional control, 293T cells were transfected with 10 μg of pHCMV-16-kDa-AU1 (18) and were lysed with Triton X-100 buffer. Cell lysates were precleared and immunoprecipitated with either anticalreticulin or anticalnexin as described above. The 16-kDa subunit of the vacuolar H^+ ATPase was examined by immunoblot assay using mouse monoclonal anti-AU (1:1,000; Babco).

GST binding assay. To verify the interaction of p12^{I} with calreticulin and calnexin, pBCp12^{I}- and pBC-transfected 293T cells were lysed with Triton X-100 buffer. One milligram of total lysate was used to bind 40 μl of a 50% slurry of glutathione-agarose beads previously blocked by 5% nonfat dry milk for 2 h. Beads were washed three times with lysis buffer and boiled in SDS sample buffer, followed by SDS-PAGE and immunoblot analysis with chicken polyclonal anticalreticulin or rabbit polyclonal anticalnexin as described above. The same membranes were stripped and tested for the expression of GSTp12^{I} or GST by immunoblot assay using rabbit polyclonal anti-GST (1:7,000).

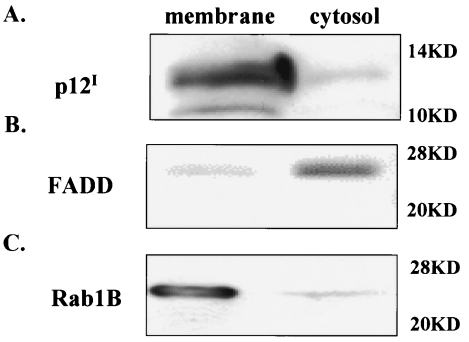

FIG. 1. p12^{I} is a membrane-associated protein. Total proteins were prepared from p12^{I}-transfected 293T cells and separated into membrane and cytosol fractions. Equal amounts of proteins from both fractions were analyzed by immunoblot assay. (A) The majority of p12^{I} was detected in the membrane fraction. FADD (B) and Rab1B (C) were used to verify the cytosol and membrane fractions, respectively. Molecular mass markers are given at right.

Glycoprotein analysis. To test whether p12^{I} is expressed as a glycoprotein, pMEp12^{I}-transfected 293T cells were lysed with RIPA buffer (1% NP-40, 1% deoxycholic acid, 10% glycerol, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5] with protease inhibitor 20-μg/ml leupeptin, 20-μg/ml aprotinin, 1 mM AEBSF, and 1 mM sodium orthovanadate). One milligram of total lysate was immunoprecipitated with rabbit polyclonal anti-HA antibody (1:150; Babco) as described above. The immune-complex-bound beads were washed three times with PBS. For the release of N-linked glycans from the protein, the beads were boiled for 5 min in 45 μl of 1× glycosidase incubation buffer (Glyco Systems) containing 0.1% SDS and 50 mM β-mercaptoethanol, followed by addition of 0.75% NP-40. The supernatant was collected and enzymatically treated overnight at 37°C using 1 mU of *N*-glycosidase F (Glyco Systems). For the release of O-linked glycans from the protein, the beads were boiled for 5 min in 45 μl of 1× *O*-glycanase reaction buffer (Sigma). The supernatant was collected and enzymatically treated at 37°C for 1 h using 10 mU of neuraminidase (Sigma), followed by overnight digestion with 1.5 mU of *O*-glycanase at 37°C. Samples were then analyzed by SDS-PAGE and p12^{I} expression, tested using an immunoblot assay as described above. The HTLV-1 surface envelope protein gp46 was used as a positive control for *N*-glycosidase F treatment. R49 cells were lysed with RIPA buffer. One milligram of total lysate was immunoprecipitated by 1:150 diluted mouse monoclonal anti-gp46 IC-11 (36). The immune-complex-bound beads were treated with *N*-glycosidase F or neuraminidase plus *O*-glycanase to release N-glycans or O-glycans by the same method described above. The expression of gp46 was tested using an immunoblot assay with IC-11.

RESULTS

p12^{I} is enriched in ER membranes. HTLV-1 p12^{I} has been demonstrated to localize in the perinuclear region and in fine reticular patterns consistent with cellular endomembranes in transfected cells (25, 26). To define the subcellular localization of p12^{I} in plasma and cellular membranes, we transiently expressed p12^{I} in 293T cells and performed membrane and cytosol fractionation. The majority of p12^{I} was detected in membrane fractions (Fig. 1A), indicating that p12^{I} is a membrane-associated protein. FADD is a cytosolic adapter protein critical for Fas signaling (27). Rab1B associates with intracellular membrane organelles and participates in the transport of glycoproteins between the ER and Golgi compartments (21).

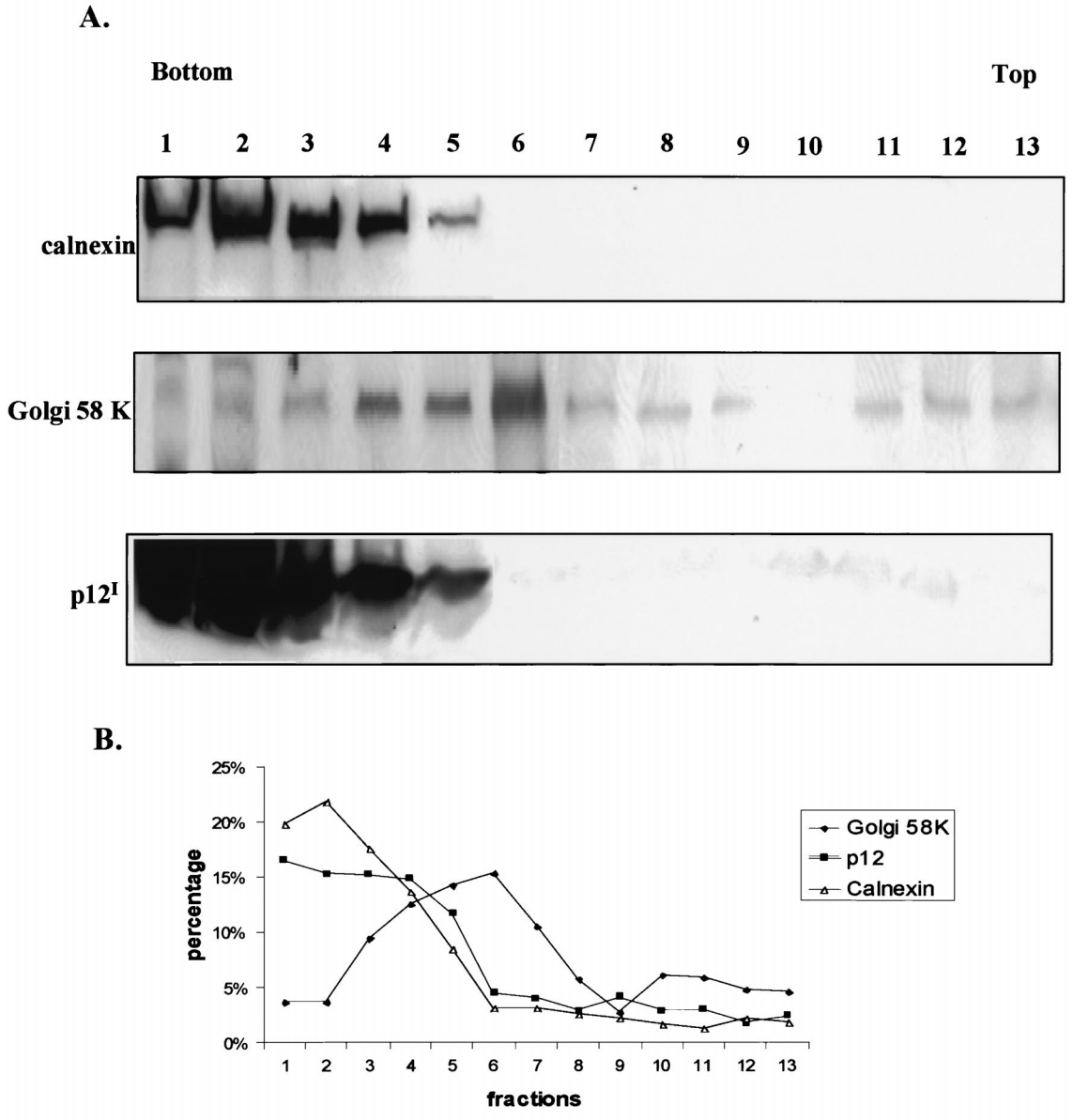

FIG. 2. p12I is enriched in ER membrane fractions. (A) The subcellular distribution of calnexin, the Golgi 58-kDa protein (Golgi 58 K), and p12I was analyzed with 293T cells transiently transfected with the pMEp12I plasmid. Following fractionation and SDS-PAGE separation, the calnexin, Golgi 58-kDa protein, and p12I were detected in an immunoblot assay by incubation with rabbit polyclonal anticalnexin, mouse monoclonal anti-Golgi 58-kDa protein, and mouse monoclonal anti-HA followed by secondary antibody incubation. The p12I and calnexin were both enriched in the ER-rich fractions 1 to 4. Numbers above the panels correspond to the fractions analyzed in panel B. (B) Graphical representation of results from subcellular fractionation of p12I-transfected 293T cells. Distribution of calnexin, the Golgi 58-kDa protein, and p12I was determined by immunoblot assay followed by densitometry image analysis. The results are expressed as percentages of the respective signals in each fraction to the sum of signals in all fractions. The ER marker calnexin was located primarily in fractions 1 to 4, and the Golgi marker Golgi 58-kDa protein was detected in fractions 4 to 12, among which fractions 4 to 7 showed enhanced expression.

These two proteins were used as markers for cytosol fractions (Fig. 1B) and membrane fractions (Fig. 1C), respectively.

To further identify the p12I-containing membrane pool, we performed gradient ultracentrifugation. Calnexin, an ER integral membrane protein, was used to identify the ER-containing fractions (Fig. 2A, fractions 1 to 5). The Golgi 58-kDa protein, a Golgi membrane resident protein, was used to identify Golgi apparatus-containing fractions (Fig. 2A, fractions 4 to 8). Fractions 4 and 5 contained both calnexin and the Golgi 58-kDa protein, consistent with the ER-to-Golgi intermediate compartment and *cis*-Golgi. The peak level of expression of p12I was detected in fractions 1 to 5, which corresponded to the calnexin-reactive ER fractions and *cis*-Golgi-containing fractions (Fig. 2).

p12I localizes in the ER and Golgi apparatus. The two major subcellular compartments in the perinuclear region are the ER and the Golgi apparatus. To further identify the subcellular compartment of p12I accumulation, multiple markers were used, including a Golgi marker (BODIPY TR ceramide), an ER marker (calreticulin), a mitochondrion marker (Mito-

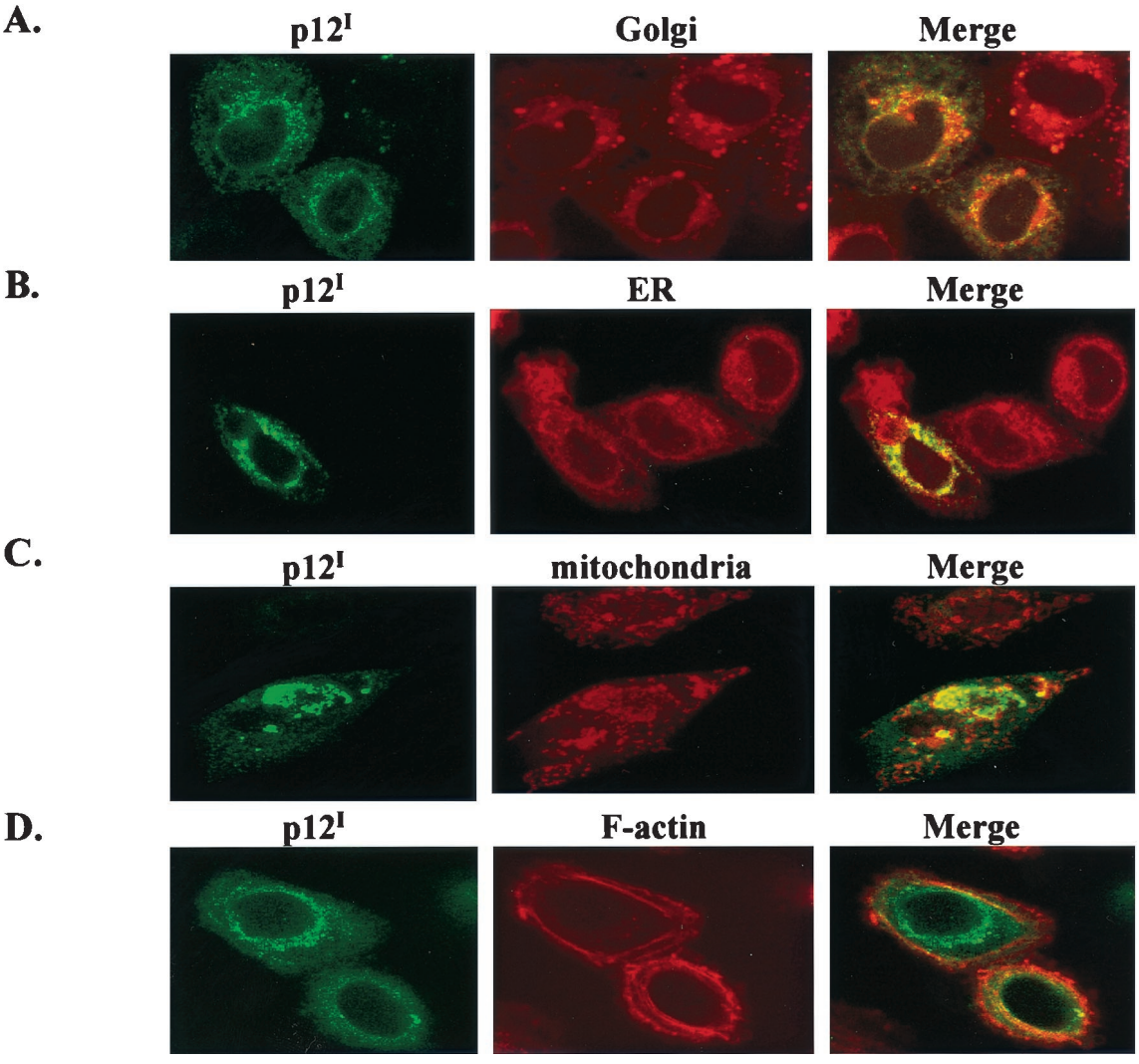

FIG. 3. p12^{I} colocalizes with the ER and Golgi apparatus. HeLa-Tat cell were transfected with pMEp12^{I}, the localization of p12^{I} was examined by indirect immunofluorescence assay, and images were collected by confocal microscopy as described in Materials and Methods. The images in the first column display the staining for p12^{I} expression. The images in the second column are the subcellular markers in the same field. The images in the third column are the merged images for p12^{I} expression and subcellular marker costaining. (A) p12^{I} colocalizes with BODIPY Ceramide (Golgi marker). (B) p12^{I} colocalizes with calreticulin (ER marker). (C) p12^{I} does not colocalize with MitoTracker Orange (mitochondria marker). (D) p12^{I} does not colocalize with rhodamine phalloidin (F-actin marker).

Tracker Orange), and an F-actin marker (rhodamine phalloidin). p12^{I} accumulation colocalized with the Golgi marker (Fig. 3A) and calreticulin (Fig. 3B), an ER luminal resident protein. However, p12^{I} accumulation did not appear to localize directly with the mitochondrial marker (Fig. 3C) or colocalize with F-actin staining (Fig. 3D). These results suggested that the accumulation of p12^{I} was distributed within the ER and the Golgi apparatus.

Electron microscopy using immunogold labeling was performed to verify the subcellular localization of p12^{I}. Gold particles corresponding to p12^{I} accumulation were localized in the membrane structures outside the nucleus, including the ER and Golgi apparatus (Fig. 4A and B) but not mitochondria (Fig. 4C). Indirect immunofluorescence and electron microscopy studies were also performed using 293 cells with similar results (data not shown).

p12^{I} localization is not sensitive to treatment with BFA. BFA is a fungal product which blocks ADP ribosylation factor activation, resulting in interference with ER-to-Golgi anterograde protein trafficking. In addition, BFA leads to the disruption of the *trans*-Golgi apparatus in treated cells (32, 43). To test whether p12^{I} localization is affected by BFA treatment, transfected HeLa-Tat cells were treated with 10 μg of BFA/ml for 30 min. The localization of p12^{I} was unchanged by BFA treatment (Fig. 5A). However, the localization of AP-1, a major component of the adapter protein complex, which is localized in the *trans*-Golgi apparatus, changed from perinuclear staining to a dispersed staining pattern following BFA treatment (Fig. 5B). These results indicate that p12^{I} accumulates in the ER or *cis*-Golgi apparatus and the localization of the protein is not ADP ribosylation factor dependent.

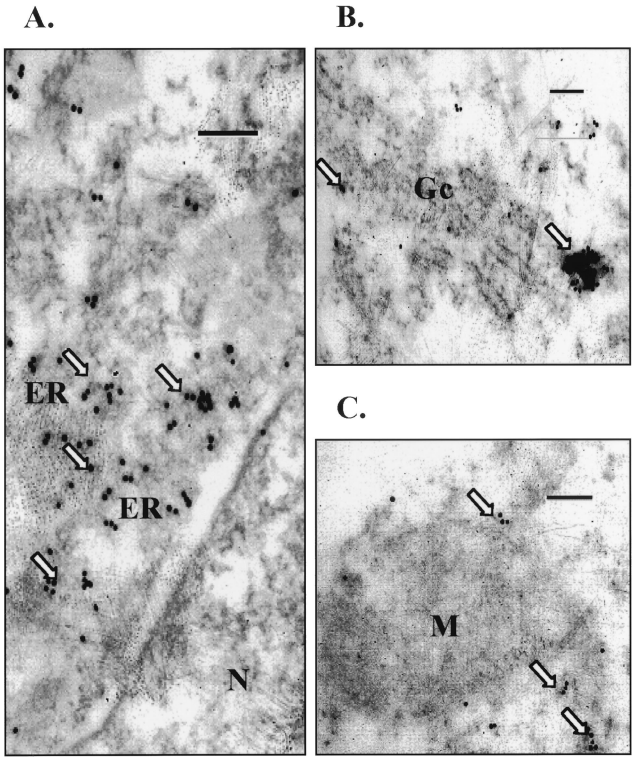

FIG. 4. p12^{I} expression identified by immunogold particles in cytoplasmic membranes. p12^{I}-transfected 293T cells were fixed, embedded, and stained as described in Materials and Methods. (A) Portion of a cell showing a nucleus (N); immunogold particles are concentrated in membrane structures outside the nucleus, consistent with the ER. (B) Portion of a cell containing Golgi stacked membranes (Gc) with accumulated gold particles. (C) Gold particles external to mitochondria (M). Magnification, ×40,500. Bar = 0.2 μm.

p12^{I} is retained in the ER. ER resident proteins maintain their localization by one of two mechanisms, retention or retrieval. To test which of these mechanisms was used by p12^{I}, we treated p12^{I}-transfected HeLa-Tat cells with cycloheximide to block new protein synthesis and then tested for p12^{I} expression. If a protein translocates from ER-Golgi compartments to the plasma membrane and then is retrieved to the ER, the protein should be transiently observed at these locations following the cycloheximide treatment. p12^{I}, however, was detected in the same perinuclear region, consistent with ER and *cis*-Golgi retention, at all time periods tested following cycloheximide treatment (Fig. 6). These data indicate that p12^{I} is retained in the ER and *cis*-Golgi compartment. Moreover, incubation with both cycloheximide and BFA did not change the p12^{I} localization, further demonstrating the retention of p12^{I} in the ER and *cis*-Golgi compartments. Since the half-life of p12^{I} has been determined to be 16 to 24 h (42), we stopped the cycloheximide treatment at 24 h postincubation. As expected, after 24 h of treatment we observed the number of p12^{I}-expressing cells to be decreased following continuous cycloheximide treatment (data not shown).

Two regions of p12^{I} (aa 1 to 47 and 48 to 99) independently determine cellular localization. The amino acid sequence of p12^{I} suggests the presence of two transmembrane domains (aa 12 to 32 and 48 to 68) within the two regions of p12^{I} that independently determine cellular localization (18). To identify regions in p12^{I} required for protein localization, eight serial deletion mutants were created (Fig. 7A). Figure 7 illustrates the serial p12^{I} deletion mutants and highlights dileucine and PXXP motifs as well as predicted transmembrane domains. The expression and correct molecular weights of wild-type p12^{I} and HA-tagged deletion mutants were confirmed by immunoblot assay (data not shown). The localization of the mutants was then examined by indirect immunofluorescence assay with transfected HeLa-Tat cells (Fig. 7B). Mutants p12^{I} 1-86 and p12^{I} 1-47 maintained the perinuclear staining typical of wild-type p12^{I}, implying that the presence of the domain in the N-terminal half of p12^{I} (aa 1 to 47) is sufficient for ER and *cis*-Golgi localization. Mutants p12^{I} 15-99, p12^{I} 32-99, and p12^{I} 48-99 also maintained the wild-type protein localization, suggesting that the C-terminal half of p12^{I} (aa 48 to 99) contained a second domain sufficient for ER and *cis*-Golgi localization. Mutants p12^{I} 15-86, p12^{I} 15-69, and p12^{I} 15-47 represented both an N-terminal 14-aa deletion and sequential deletions in the C terminus of the protein. The mutated proteins 12^{I} 15-86 and p12^{I} 15-69 maintained the wild-type p12^{I} pattern of localization. However, p12^{I} 15-47 was diffusely expressed throughout the cell. Taken together, these data suggest that either of the two protein regions (aa 1 to 47 or 48 to 99) previously predicted to contain putative transmembrane domains (aa 12 to 32 and 48 to 68, respectively) (18) is sufficient for the viral protein to maintain ER and *cis*-Golgi localization. In addition, our data indicate that the N-terminal 14 aa of the protein are critical for the first transmembrane region to maintain the wild-type protein accumulation pattern.

p12^{I} is associated with calreticulin and calnexin. Calreticulin and calnexin, ER resident proteins, bind to a variety of viral glycoproteins, including hepatitis C virus (HCV) E1 and E2 (8), human immunodeficiency virus type 1 envelope gp160

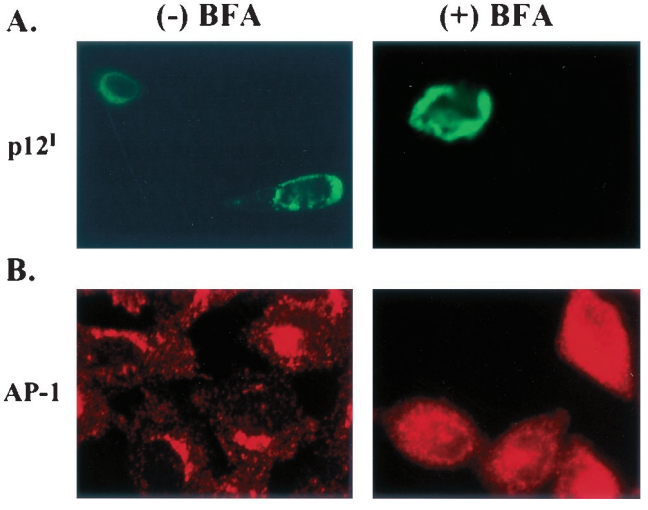

FIG. 5. p12^{I} localization is not sensitive to BFA treatment. GFPp12^{I}-transfected HeLa-Tat cells were treated with BFA (10 μg/ml) (right column) for 30 min or left untreated (left column). Cells were fixed and stained using a monoclonal antibody against AP-1, followed by Cy3-labeled anti-mouse antibody staining. (A) p12 localization did not change following BFA treatment. (B) AP-1 staining became diffuse following BFA treatment.

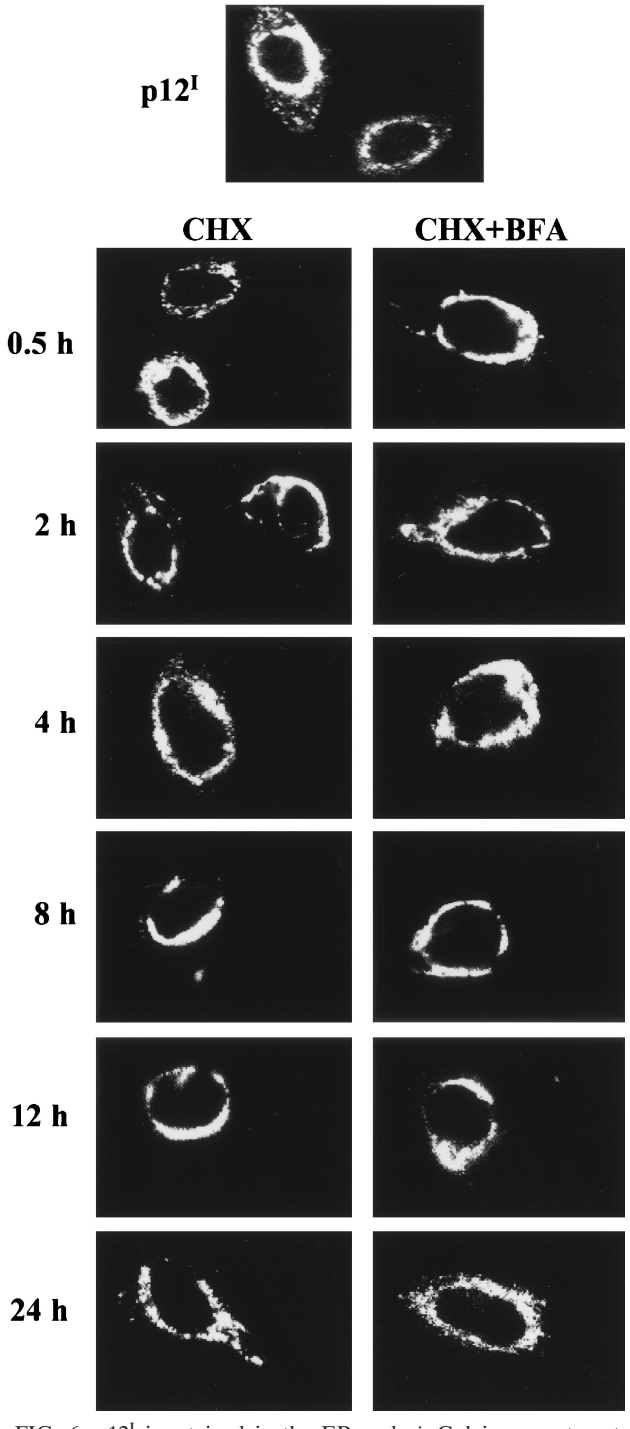

FIG. 6. p12^{I} is retained in the ER and *cis*-Golgi compartments. HeLa-Tat cells were transfected with pMEp12^{I} and were treated with cycloheximide (CHX) at 24 h posttransfection. The localization of p12^{I} was examined at various time points following cycloheximide treatment by indirect immunofluorescence assay. The perinuclear staining pattern did not change following continuous cycloheximide treatment and BFA treatment (CHX+BFA).

(35), and human cytomegalovirus glycoprotein B (48) to promote protein folding. Among these, the HCV E1 and E2 proteins are retained in the ER. The fact that p12^{I} colocalizes with calreticulin and calnexin in the ER implied that calreticulin and calnexin, as molecular chaperones, may bind to p12^{I}. To address this question, we performed both coimmunoprecipitation and GST binding assays. Our data indicated that polyclonal anticalnexin and anticalreticulin coprecipitated p12^{I} from transfected cells (Fig. 8A and B). Additionally, GSTp12^{I} was able to pull down calnexin and calreticulin in the GST binding assay (Fig. 8C and 8D). The expressions of calnexin, calreticulin, GSTp12^{I}, and GST were tested in Western blots (lower panels in Fig. 8). Normal rabbit serum did not precipitate p12^{I} (Fig. 8A and B). The GST vector alone did not bind to either calnexin or calreticulin (Fig. 8C and D). In addition, we tested the possible association between another membrane-associated protein, the 16-kDa subunit of the vacuolar H^{+} ATPase, and calreticulin or calnexin. The 16-kDa subunit protein exhibited moderate binding to calnexin but failed to bind calreticulin (data not shown).

Deglycosylation analysis of p12^{I}. Both calreticulin and calnexin serve as ER chaperones and predominantly bind to N-linked glycoproteins to promote their folding. Therefore, we performed deglycosylation analysis of p12^{I} to test whether p12^{I} is a glycoprotein. *N*-glycosidase F or neuraminidase plus *O*-glycanase were used to enzymatically digest immunoprecipitated p12^{I} to release possible N-linked glycans from asparagine or possible Galβ1-3GalNAc from serine or threonine residues in the protein. The electrophoretic mobility of p12^{I} did not change following treatment with either *N*-glycoside F or *O*-glycanase (Fig. 9A), indicating that p12^{I} is not a glycosylated protein. The N-linked glycoprotein HTLV-1 gp46, which migrated faster following treatment with *N*-glycosidase (Fig. 9B), served as a positive control for *N*-glycosidase F digestion. Taken together, our data are the first to show that a nonglycosylated viral protein colocalizes with and specifically binds to calreticulin and calnexin in the ER. Calreticulin and calnexin also function to modulate calcium storage in the ER. Taken together, our data suggest a role for p12^{I} in cell activation through calcium-mediated cell signaling initiated from the ER.

DISCUSSION

In this study, we determined the subcellular accumulation of HTLV-1 p12^{I} to further define the possible function for this protein in viral infection. We demonstrate that p12^{I} is retained as a membrane-associated protein and is expressed predominantly in the ER and *cis*-Golgi apparatus. Two regions of the protein containing predicted transmembrane domains are independently responsible for this pattern of localization. Importantly, we are the first to identify the interaction of p12^{I} with both calreticulin and calnexin in the ER, suggesting a possible function of the viral protein in calcium-mediated cell signaling leading to host cell activation. These findings are consistent with our previous studies that demonstrated a requirement for HTLV-1 p12^{I} in viral infectivity both in a rabbit model of infection (12), in primary lymphocytes in vitro (1), and in calcium-dependent nuclear factor of activated T cells-mediated transcription (B. Albrecht et al., unpublished data).

HTLV-1 p12^{I} is highly hydrophobic and has been shown to localize to the perinuclear region (25, 26). Consistent with these previous reports, we biochemically confirmed, using cell fractionation, that p12^{I} was associated with cellular membranes. Since the ER and the Golgi apparatus are two major

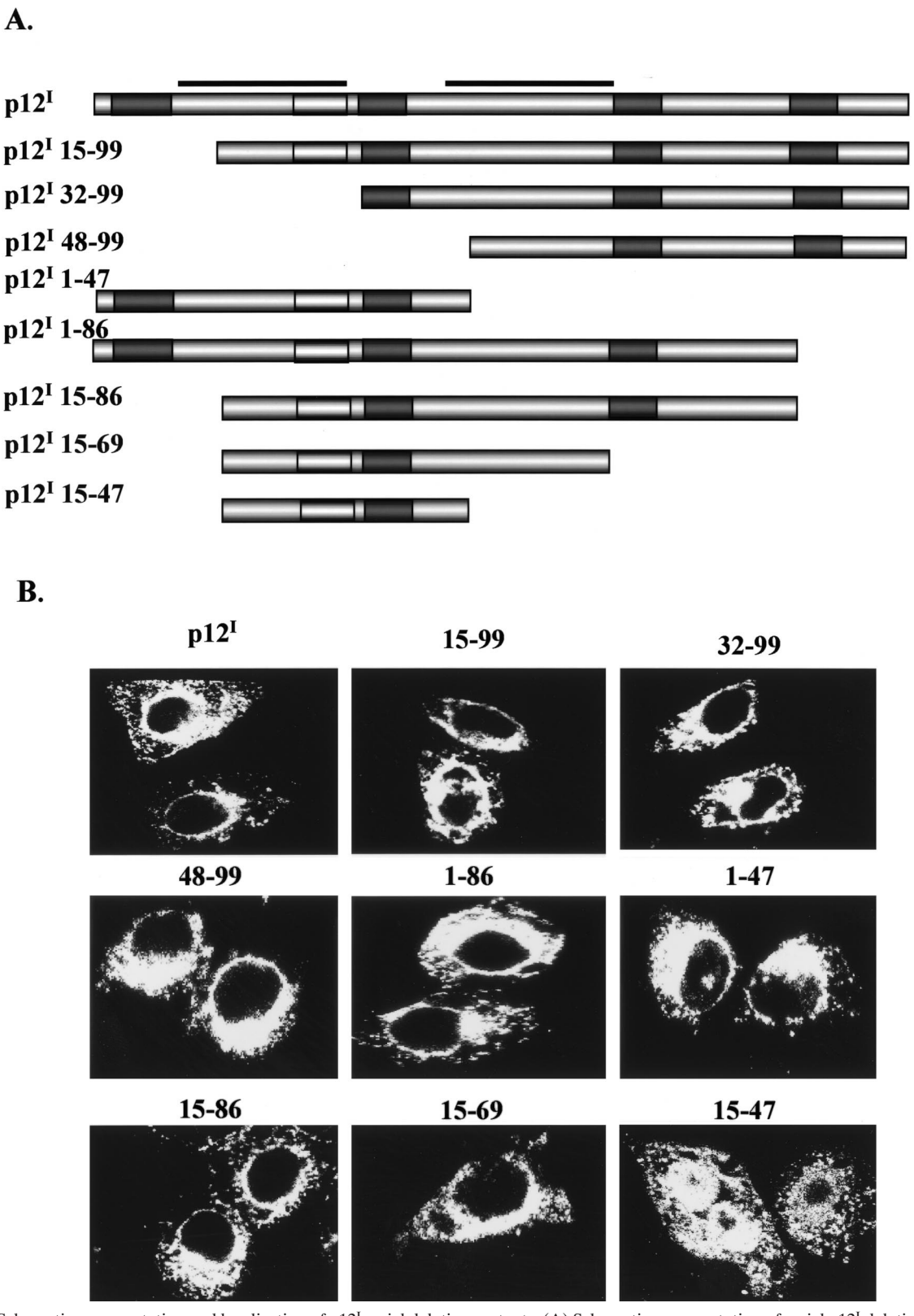

FIG. 7. Schematic representation and localization of p12^{I} serial deletion mutants. (A) Schematic representation of serial p12^{I} deletion mutants. Open box, dileucine motif; shaded box, PXXP motif; bar, transmembrane domain. (B) Localization of wild-type p12^{I} and serial deletion p12^{I} mutants. The images were examined at 48 h posttransfection by indirect immunofluorescence assay. The N-terminal deletion mutants p12^{I} 15-99, p12^{I} 32-99, and p12^{I} 48-99 maintained the perinuclear staining typical of wild-type p12^{I}. C-terminal deletion mutants p12^{I} 1-86, p12^{I} 1-47, and dual deletion mutants p12^{I} 15-86 and p12^{I} 15-69 also displayed perinuclear staining. The last mutant, p12^{I} 15-47, lost the perinuclear staining pattern and was diffusely expressed.

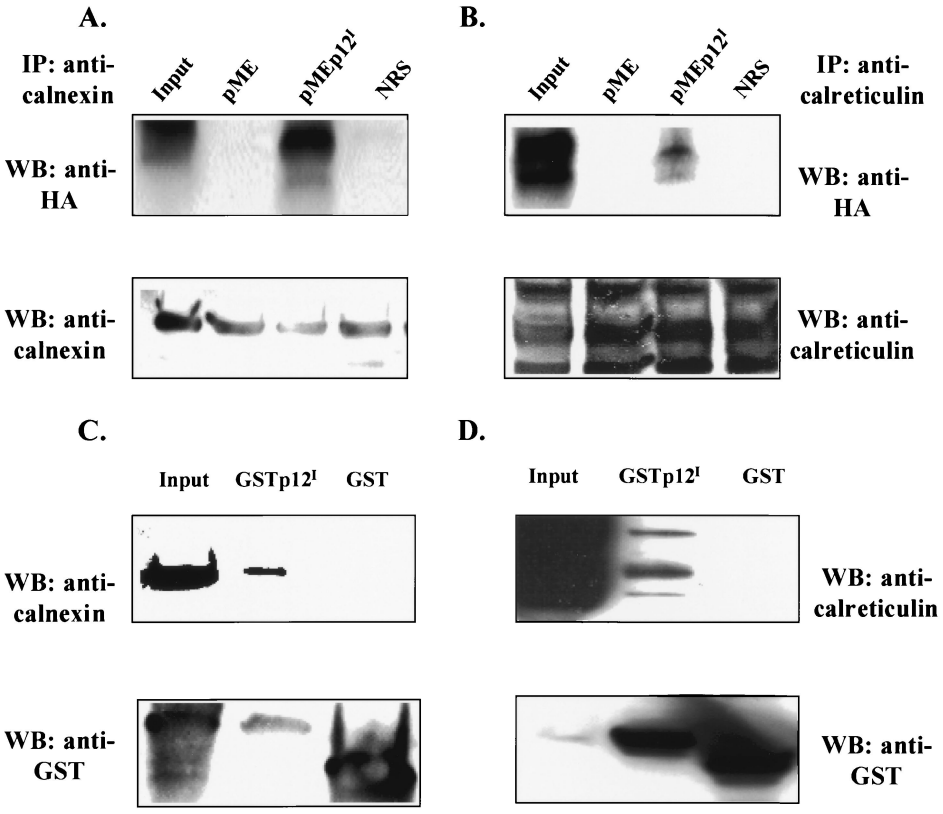

FIG. 8. Association of p12^{I} with calreticulin and calnexin. (A and B) Coimmunoprecipitation of p12^{I} with calreticulin or calnexin. p12^{I}- and vector-transfected 293T cell lysates were immunoprecipitated with rabbit polyclonal anticalnexin (A) or anticalreticulin (B), followed by SDS-PAGE separation and Western blot analysis using mouse monoclonal anti-HA. NRS, normal rabbit serum. The expression of calreticulin or calnexin in each lysate was concurrently tested by immunoblot assay using rabbit polyclonal anticalnexin (A, lower panel) or chicken polyclonal anticalreticulin (B, lower panel). (C and D) GSTp12^{I} binds to calreticulin and calnexin. GSTp12^{I}- and GST-transfected 293T cell lysates were incubated with glutathione-agarose beads, followed by SDS-PAGE and Western blot analysis using rabbit polyclonal anticalnexin or chicken polyclonal anticalreticulin. The membrane was stripped and was tested for the expression of GSTp12^{I} and GST.

organelles present in the perinuclear region, it was likely that p12^{I} was localized to these two compartments. We costained p12^{I} with various subcellular markers to more specifically identify the accumulation pattern of p12^{I} in these compartments. p12^{I} colocalized with both calreticulin, an ER resident protein, and the Golgi apparatus. These findings were further verified by immunoelectron microscopy. Thus, our data indicate that p12^{I} accumulation is confined principally to the ER and *cis*-Golgi apparatus. This tenet was further confirmed by our studies using BFA, which acts to block ADP ribosylation factor-GTP formation and therefore disrupts ER-to-Golgi protein transport. The localization of a *trans*-Golgi protein, AP-1, was disrupted by BFA treatment. In contrast, the accumulation pattern of p12^{I} was unchanged by BFA treatment, suggesting that the ER and *cis*-Golgi apparatus are the major compartments in which the viral protein accumulates. These data were confirmed by our subcellular fractionation study. Importantly, we used three different expression plasmids, including pMEp12^{I}, GFPp12^{I}, and GSTp12^{I}, to investigate the localization of p12^{I}. Although these plasmids contain different promoters and vary in their level of expression of the tagged protein (25), they all exhibited identical patterns of p12^{I} accumulation, indicating the strong tendency of the protein to be retained in the ER and *cis*-Golgi compartments.

In general, proteins that are expressed in the ER achieve their specific localization in two different but potential overlapping mechanisms: by direct retention or by retrieval from cellular compartments through recognition of specific cellular motifs. To test whether p12^{I} maintained its localization by retention or retrieval, we used cycloheximide to block protein synthesis and tested for the expression of the protein over a 24-h period. The typical perinuclear localization of p12^{I} did not change following either cycloheximide treatment alone or cotreatment with cycloheximide plus BFA, indicating that p12^{I} is retained in the ER and *cis*-Golgi compartment. Among the most extensively studied ER localization signals is a C-terminal KDEL amino acid sequence (20), which is responsible for the localization of calreticulin to the ER (28). Two well-characterized motifs, the C-terminal KKXX sequence and the N-terminal double arginine (RR) motif, are sufficient to retain type I integral membrane protein (amino terminus in the lumen) and type II integral membrane protein (C terminus in the lumen) to the ER, respectively (20). The amino acid sequence of p12^{I} does not contain a KDEL, KKXX, or RR motif. Therefore, the ER retention of p12^{I} is likely related to other structural features of the protein.

To map the region required for the localization of p12^{I} to the ER, we sequentially deleted both the amino terminus and

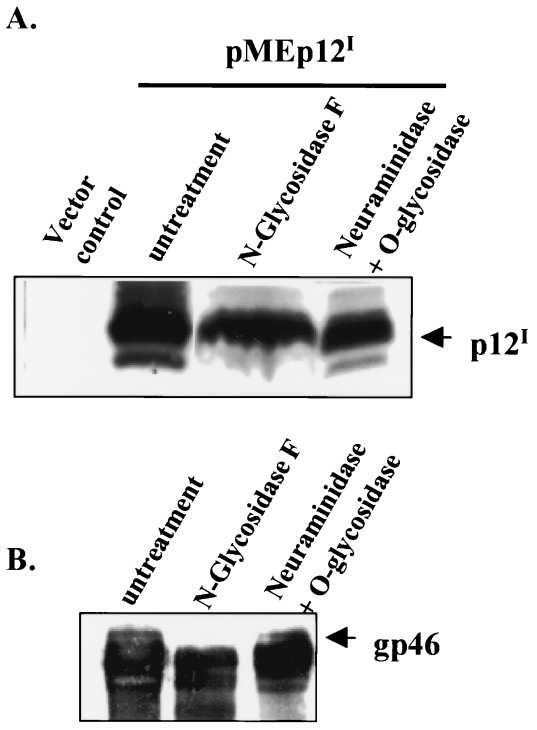

FIG. 9. p12^{I} is not a glycoprotein. (A) p12^{I}-transfected 293T cell lysates were immunoprecipitated by rabbit polyclonal anti-HA. The immune complex-bound beads were treated with either *N*-glycosidase F or neuraminidase plus *O*-glycanase overnight. The beads were boiled in SDS sample buffer, and the proteins were separated by SDS-PAGE. Expression of p12^{I} was tested by mouse monoclonal anti-HA. (B) R49 cells, rabbit peripheral blood mononuclear cells transformed by an HTLV-1 molecular clone (ACH), were lysed and immunoprecipitated by IC11 antibody (mouse monoclonal anti-gp46). The immune-complex-bound beads were treated with either *N*-glycosidase F or neuraminidase plus *O*-glycanase overnight. The beads were boiled in SDS sample buffer, and the proteins were separated by SDS-PAGE. The expressions of HTLV-1 envelope (gp46) were tested by IC11.

the carboxyl terminus of p12^{I}. Interestingly, either N-terminal mutants (p12^{I} 15-99, p12^{I} 32-99, p12^{I} 48-99) or C-terminal mutants (p12^{I} 1-86, p12^{I} 1-47) alone maintained patterns of staining typical of the full-length protein. A computer analysis of the amino acid sequence of p12^{I} predicted the presence of two transmembrane domains (aa 12 to 32 and 48 to 68) within these two regions (18). Similar to the findings of Koralnik et al. (26), our data indicated that two regions in p12^{I} (aa 1 to 47 and 48 to 99) are independently sufficient for the localization. In this previous study, however, deletion of the first 12 aa from the N-terminal region of the protein did not influence the perinuclear accumulation of the protein. Our results indicate that deletion of the first N-terminal 14 aa (mutant p12^{I} 15-47) resulted in a loss of perinuclear localization. Taken together, these data indicate the importance of aa 12 to 14 in the function of the first transmembrane domain of p12^{I}. Ongoing work in our laboratory seeks to further define critical motifs of the protein in both localization and functional studies (1). Our data are consistent with studies which indicate the importance of transmembrane domains in the ER localization for both HCV E1 (11) and rubella virus E1 (22).

Calreticulin and calnexin each have been demonstrated to modulate calcium storage and control protein folding, including that of several viral glycoproteins, in the ER (28, 31). Our data indicate that p12^{I} binds to each of these ER resident proteins. Within the ER, p12^{I} may serve to modulate calcium-mediated signals involved in cell activation. Our parallel studies indicate that expression of p12^{I} in Jurkat T cells enhances reporter gene activity mediated by the nuclear factor of activated T cells in a calcium-dependent manner (Albrecht et al., unpublished). Alternatively, these proteins may serve as molecular chaperones to regulate the folding of p12^{I}. As molecular chaperones, both calreticulin and calnexin have been predominantly shown to bind N-linked glycoproteins. Asparagine at aa 51 of p12^{I} is a possible N-linked glycosylation site for the viral protein. However, our deglycosylation analysis revealed neither N-linked glycosylation nor O-linked glycosylation in p12^{I}. Further studies will be required to determine the possible role of p12^{I} in calcium storage and release from the ER. Interestingly, Johnson et al. (23) have reported that p12^{I} binds to the heavy chain of major histocompatibility complex (MHC) class I and prevents its association with β_2-microglobulin, impairing the traffic of the protein complex. Calreticulin also acts as a chaperone in the assembly and expression of MHC class I molecules in activated human T lymphocytes (2). One potential mechanism to explain the ability of p12^{I} to interfere with MHC class I complex transport is binding and retaining of calreticulin-MHC class I complexes in the ER or *cis*-Golgi.

In summary, our data illustrate that p12^{I} is accumulated and retained in the ER and *cis*-Golgi apparatus. We have determined that two regions in p12^{I} are independently sufficient for p12^{I} localization, and we are the first to identify the association between a nonglycosylated viral protein, p12^{I}, and resident ER proteins, suggesting a role of p12^{I} in calcium-mediated signals during cell activation. Our data support emerging evidence for the role of p12^{I} in early events of T-cell signaling to enhance the replicative ability of HTLV-1.

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